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E. Fabiani
Institut Laue-Langevin, Grenoble

A. M. Stadler
Institut Laue-Langevin, Grenoble

D. Madern
Institut de Biologie Structurale Grenoble

M. M. Koza
Institut Laue-Langevin, Grenoble

M. Tehei
University of Wollongong, moeava@uow.edu.au

See next page for additional authors

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Abstract

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Keywords

Amyloid former, Protein dynamics, Neutron, Circular dichroism

Disciplines

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Authors

E. Fabiani, A. M. Stadler, D. Madern, M. M. Koza, M. Tehei, M. Hirai, and G. Zaccai

DYNAMICS OF APOMYOGLOBIN IN THE ALPHA-TO-BETA TRANSITION AND OF PARTIALLY UNFOLDED AGGREGATED PROTEIN

E. Fabiani [1,2,3,4,*], A.M. Stadler [1,5], D. Madern[2], M.M. Koza[1], M. Tehei[1,6,7] M. Hirai[8], G. Zaccai[1,**]

[1] *Institut Laue-Langevin BP156, Grenoble (France)*

[2] *Institut de Biologie Structurale Grenoble (France)*

[3] *Université Joseph Fourier, Grenoble (France)*

[4] *present address : CEA LETI Minatec Département des Technologies pour la Biologie et la Santé, Grenoble Cedex 9, France*

[5] *Research Centre Juelich, Juelich (Germany)*

[6] *Present address: School of chemistry-University of Wollongong-NSW 2522 (Australia)*

[7] *Present address: Australian Institute of Nuclear Science and Engineering (AINSE), Menai (Australia)*

[8] *Gunma University (Japan)*

*Corresponding author during the submission:

Dr. E. Fabiani (elisa.fabiani@cea.fr) telephone: +33 438 783910

**Corresponding author after the publication:

Dr. G. Zaccai (zaccai@ill.fr) telephone: +33 476 207679

Abstract

Changes of molecular dynamics in the α-to-β transition associated with amyloid fibril formation were explored on apo-myoglobin (ApoMb) as a model system. Circular dichroism, neutron and X-ray scattering experiments were performed as a function of temperature on the protein, at different solvent conditions. A significant change in molecular dynamics was observed at the α-to-β transition at about 55 ˚C, indicating a more *resilient* high temperature β structure phase. A similar effect at approximately the same temperature was observed in holo-myoglobin, associated with partial unfolding and protein aggregation. A study in a wide temperature range between 20 K and 360 K revealed that a dynamical transition at about 200 K for motions in the 50 ps time scale exists also for a hydrated powder of heat-denatured aggregated ApoMb.

Introduction

Amyloidosis is an emerging category of diseases characterised by the extracellular accumulation of protein aggregates in body organs or tissues, including brain, liver, spinal cord, and intestine. Even if the first cases of amyloidosis were described over 300 years ago, it is only within the past 20 years that the specific chemical composition and structure of amyloid protein formations have been understood. More than twenty different kinds of amyloidosis are known currently. They include Alzheimer's disease, Parkinson's disease, Huntington's disease, and the "prion diseases" (Scrapie, Kuru, CJD, BSE, etc.). Therapeutic approaches have focused on reducing the production of the protein, with different treatments for the different cases of amyloidosis.

Amyloid aggregation is closely related to protein folding issues. "Amyloid fibrils" consist of polymerised cross-beta-sheet structures in which the beta-strands are arranged perpendicular to the long axis of the fibre. There are various causes for protein misfolding that could lead to amyloid formation. For example, in the absence of chaperones, certain proteins will fail to achieve their native state and may associate with other unfolded polypeptide chains to form large amyloid fibrils. Misfolding can also occur when a protein is subjected to particular conditions, such as extremes of heat or pH. Understanding the physical bases of misfolding in these cases is of fundamental scientific and biotechnological importance. In the context it is important to characterise the forces that stabilise protein structure, and therefore its dynamics under relevant solvent conditions.

Apo-myoglobin (ApoMb) was chosen for neutron, X-ray scattering and circular dichroism measurements because previous studies on the protein have shown that its fibril formation appears under particular conditions of temperature and pH (Fändrich et

al., 2003). Before amyloid formation, ApoMb can adopt two well-defined structural conformations at pH 9: below 55 ^{o}C, the *helix-rich native-like structure α*, and above 55 ^{o}C, *the cross-β structure* characteristic of proteins that can generate amyloid deposits in humans. The transition arises because the helical structures in ApoMb are destabilised partially so that neighbouring strands will interact to form the cross-β structure between them. Amyloid structures differ from globular protein structures, which are always encoded in the amino acid sequence, because they do not depend on the presence of distinctive sequence patterns or specific intramolecular side-chain interactions. The structural aspects of the transition have been characterised by small and wide angle X-ray scattering (Onai et al., 2007). The total structure factor observed showed two peaks at 0.58 and 1.34 $Å^{-1}$, which are strongly correlated with amyloid transformation (Onai et al., 2007). The peaks appear at pH 9 at 55 $^{\circ}$C, and become more pronounced as the temperature is raised. Further investigations of structure (Booth 1997, Chamberlain 2001) and folding dynamics (Canet et al., 1999, Canet et al., 2002) have led to a better understanding of the process involved in the conversion of globular proteins into amyloid fibrils.

The work reported concerns the measurement and comparison of the structure and dynamics, in a wide temperature range, of ApoMb under different solvent conditions (at pH 9 and pH 7, in H_2O and D_2O), by using circular dichroism (CD) to characterise secondary structures, and energy resolved elastic neutron scattering. As it has been demonstrated for other biological systems, energy resolved neutron scattering provides quantitative information on the forces involved in the stabilisation of protein structures (Zaccai 2000). The scattering cross-section of ^{1}H dominates that of all other atoms in biological material, and of its isotope, deuterium ^{2}H (D)**.** Thus, heavy water (D_2O) is often used to reduce the contribution of hydration water to the scattering signal. CD

experiments were performed in H_2O and D_2O, therefore, to characterise whether or not the α-to-β transition is affected by the solvent isotope.

A significant change in molecular dynamics towards a more resilient structure was observed for ApoMb at pH 9, at the α-to-β transition at about 55 ˚C. A similar observation for holo-myoglobin was attributed to partial unfolding and protein aggregation, indicating that denatured and aggregated protein display similar dynamics as β-amyloid. In a further examination of the dynamics of irreversibly heat-denatured and aggregated protein, a hydrated powder sample of ApoMb was shown to undergo a dynamical transition at about 200 K as has been observed for native holo-myoglobin (Doster et al., 1989), purple membranes (Ferrand et al., 1993, Lehnert et al., 1998).

Materials and Methods

ApoMb preparation

ApoMb was obtained by removing the heme from holo-myoglobin. Several protocols yield the apo-protein with similar properties. We successfully adapted the method of Rothgeb (Rothgeb and Gurd, 1978) to obtain the apo-protein form in gram quantities for the neutron scattering experiments. The process was promoted by acid conditions in suitable solvent. An appropriate amount of lyophilised horse heart holo-myoglobin purchased from SIGMA-ALDRICH was dissolved in water. The pH of the holo-myoglobin solution was then lowered to 1.5 with concentrated HCl at 4 °C. The acidified solution was extracted as quickly as possible with 4 volumes of 2-butanone. The upper organic layer was decanted, and the extraction was repeated at least twice more in order to obtain a hazy, colourless aqueous layer which was dialysed exhaustively against a dilute bicarbonate solution followed by pure water. After salt was completely removed by dialysis, the protein was lyophilised. For the neutron experiments, the powder was hydrated by pipetting uniformly buffer solutions to the

sample to a level of 0.73g H_2O/ 1g protein or corresponding 0.8g D_2O/ 1g protein. This corresponds to approximately two hydration layers per protein and allows for pH effects. CD experiments were performed on the protein solution before lyophilisation.

Solvent conditions

For neutron experiments, the lyophilised ApoMb was re-hydrated under different solvent conditions in H_2O and D_2O potassium phosphate buffer (20mM KH_2PO_4, pH/pD 7 and pH/pD 9). Potassium phosphate was purchased from SIGMA.

All solutions were obtained by dissolving the buffer in distilled water (H_2O) or heavy water (D_2O). The pH (pD) was adjusted to the desired value by adding acid or base. The pD was calculated by adding 0.4 to the value measured on a normally calibrated pH metre (Lide 1999). The same buffer solution was used to adjust the pH (pD) of the protein solution for the CD experiments.

Circular Dichroism (CD) experiments

Circular dichroism (CD) measurements were used to check the α-to-β transition, the pH (pD) and temperature dependence of secondary structure. CD spectroscopy measures differences in the absorption of left-handed polarized light versus right-handed polarized light, which arise due to structural asymmetry. The absence of regular structure results in zero CD intensity, while an ordered structure results in a spectrum, which can contain both positive and negative signals. CD spectroscopy is particularly good for determining whether or not a protein is folded, and if so characterizing its secondary structure. CD measurements were performed at the Institut de Biologie Structurale (IBS) and EMBL in Grenoble (France) by using a CD spectrometer equipped with a temperature-controlled cuvette holder. The far-UV CD spectral region (190-250 nm) was explored for different temperatures, pH and buffer conditions. The protein concentration was about 20 μM for all samples. The experiments were

performed in the temperature range between 25 and 95 °C. The data were analysed by subtracting the background CD spectrum and converting the spectra from millidegrees to molar ellipticity (θ) in degrees·cm^2 per decimole residues using the measured protein concentration. Secondary structure analysis of the final CD data was carried out using the K2D web program (Andrade et al., 1993, Merelo et al., 1994).

Neutron scattering experiments

Neutron scattering has proven to be a versatile tool for the study of the molecular dynamics of condensed matter in general, and biological macromolecules and solvent interactions, in particular (Gabel et al., 2002, Paciaroni et al., 2002, Lehnert et al, 1998, Gabel and Bellisent-Funel, 2007, Andreani et al., 1995, Pieper et al., 2004). The complexity of protein dynamics is reflected in a vast range of types of motion and corresponding associated time and length scales. Neutrons are sensitive in different ways to the motions of hydrogen (H) and its heavy isotope deuterium (D). Isotope labelling as well as the use of spectrometers with different energy resolution (corresponding to different time scales) considerably enriches the interpretation of scattering spectra. Elastic incoherent neutron scattering (EINS) is a specific technique for the study of dynamical processes occurring on a time scale defined by the spectrometer resolution in energy. It provides atomic mean square displacement values (dominated by H atoms because of their large scattering cross-section compared to all the other atoms in biological samples) as a function of temperature, from which effective force constants can be obtained (Zaccai 2000).

The mean square displacement $<u^2>$ is derived from the analysis of the Elastic Area *vs* Q^2 in a Gaussian approximation, where the Elastic Area is the area of the elastically scattered intensity and Q is the modulus of the scattering vector, $(4\pi \sin \theta/2)/\lambda$ (Gabel et al., 2002) where θ is the scattering angle and λ is the neutron wavelength.

$$< u^2 > = -6\left(\frac{d\, ln[Elastic\ Area]}{d(Q^2)} \right) \quad (1)$$

The approximation is valid for values of $<u^2> Q^2$ smaller than or close to about 2, and the Q ranges for the fit were chosen appropriately.

In cases for which the data quality is not sufficient to derive precise mean square displacement values, an analysis as a function of temperature of the total intensity sum in the same Q range, can give a good indication of a transition in dynamical behaviour (Reat et al., 2000).

- At the Institut Laue-Langevin reactor neutron source -

Energy resolved elastic neutron scattering experiments on ApoMb and HoloMb were carried out at the Institut Laue-Langevin in Grenoble (France) on the spectrometer IN13 (http://www.ill.eu/YellowBook/IN13/). IN13 is a back-scattering spectrometer characterised by a high-energy resolution (of about 8 μeV, corresponding to 0.1 ns time-scale) associated to a wide range of momentum transfer (Q_{max} ~ 4.7 $Å^{-1}$, corresponding to a length scale ~ Ångstrom unit). Free and hydration water motions are too fast to be observed in this time-length window for q>1 $Å^{-1}$ and contributes to the measured signal only as a negligibly low background. Measured data was analyzed in the q^2-range from 1.5-5 $Å^{-2}$. In this high scattering vector range experiments in natural abundance water solvent are feasible with negligible H_2O contribution (Tehei et al., 2004, Jasnin et al., 2008). An amount of 100 mg and 120 mg of re-hydrated ApoMb and HoloMb powder, respectively, (0.73 g H_2O/ 1g protein) at pH 9 was inserted in a flat aluminium cell with internal spacing of 0.3 mm, which was put in the IN13 sample environment at an angle of 135^o with respect to the incident beam. The experiments were performed as a function of temperature (heating between 280 K and 350 K). An displex device specific to IN13 was used for termperature control. The data were analysed as outlined above.

- At the ISIS spallation neutron source -

Neutron scattering experiments on a heat-denatured and aggregated ApoMb hydrated powder sample were carried out on the IRIS spectrometer at the ISIS spallation neutron source, Chilton, UK. The denatured sample was obtained by heating the hydrated powder in a closed sample holder at 360 K and allowing incubation for 4 h. By exploiting the Pyrolitic Graphite PG(002) analyser configuration and the Beryllium filter, the covered momentum transfer range was between 0.3 and 1.8 $Å^{-1}$ with an energy resolution of 17 μeV (corresponding to a 50 ps time scale). The experiment was performed with D_2O solvent, since diffusion of H_2O would contribute strongly to the measured signal at the small scattering vector range covered by the instrument (Jasnin et al., 2008. An amount of 130 mg hydrated powder (0.8 g D_2O/ 1g protein) at pH 9 in D_2O was held in a flat aluminium cell with internal spacing of 0.3 mm, placed at an angle of 135^o with respect to the incident beam. We estimated that D_2O solvent contributes only with 2% to the total incoherent scattering cross section. Therefore, it is justified to neglect the solvent contribution of D_2O. Each temperature point was collected for 4 hours. The temperature scan was performed in a temperature range between 20 K and 360 K in order to examine the mean square atomic displacements and effective force constants (Zaccai 2000). It was reported in literature that water in the secondary hydration shell of proteins can be vitrified during flash cooling to 100 K and crystallizes during reheating at 200-210 K to cubic ice (Sartor et al., 1995). Even if there was a secondary hydration shell present in our sample, we didn't observe ice formation during the experiment at low temperature as evidenced by the absence of ice Bragg peaks. We can only speculate about the reason, but the presence of buffer solution and not pure water might be an explation. The temperature was controlled by heating the sample in a standard IRIS cryofurnace. First the sample was inserted at 280 K. Then it was heated up to 360 K to allow for heat denaturation. Afterwards the

sample was cooled down to 20 K and the scattering at low temperatures was measured as a reference. In the following, the temperature scan was measured for different temperature points from 20 K to 360 K. Heating and cooling rates were approximately in the order of 1-3 K/min. Mean square displacements were calculated from the analysis of the integrated intensity over a frequency window corresponding to instrumental resolution around the elastic peak, according to equation 1.

Wide-angle X-ray scattering experiments

To check an effect of change of solvent (H_2O or D_2O) on α-to-β transition and amyloid transition of ApoMb, we have carried out wide-angle X-ray scattering (WAXS) measurement under the same solvent condition used before (Onai et al., 2007), where the D_2O solvent was 50 mM Tris–DCl (2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride) buffer at pD 9. The ApoMb concentration was 1 % w/v. The WAXS measurement was performed by using the BL-40B2 spectrometer installed at the synchrotron radiation source (SPring-8) at the Japan Synchrotron Radiation Research Institute (JASRI), Japan. The X-ray wavelength, the sample-to-detector distance and the exposure time were 0.82 Å, 41 cm and 30 s, respectively. The details of the scattering data analysis were as reported previously (Hirai et al, 2004).

Results and Discussion

Circular Dichroism

The far-UV CD spectra of ApoMb are presented in Figure 1 for all temperatures and solvent conditions explored. At room temperature, all spectra show two negative peaks at 207 and 222 nm and a positive one at 193 nm, characteristic of the alpha-helix structure (black curves). ApoMb shows the predominance of the alpha-helix structure

until 55 °C. Above 55 °C and only at pH and pD 9, the beta-sheet contribution appears and replaces the alpha-helix secondary structure. This is evident in Figure 1 B) and 1 D) by the shift of the minimum from 207 nm to at around 200 nm during heating (Faendrich et al., 2003) and by the behaviour of the beta sheet content from the K2D analysis of the spectra shown in Figure 2. Note that the K2D data show similar behaviour in H_2O and D_2O at corresponding pH or pD values, respectively. Synchrotron radiation experiments also showed similar behaviour at the large angle scattering features (indicative of secondary structure) irrespective of whether the sample was in H_2O or D_2O as shown below.

In Figure 1 A) and 1 B), at pD (pH) 7 in D_2O and H_2O, the amplitudes of the two peaks are lowered with increasing temperature, consistent with the expected partial unfolding of the protein. Analysis of the spectra with the program K2D (Figure 2) yielded helix fractions of 70-80 % and 40% at 20 ºC and 70 ºC, respectively, while the beta fraction, which was negligible at room temperature increased to about 15 % at high temperatures. A random fraction increased from 20-30 % to 40-60 % between 20 ºC and 70 ºC. However, in Figure 2 at pH (pD) 9 in H_2O and D_2O, the helix fraction decreases rapidly to 15-25% between 20 ºC and 70 ºC and the beta fraction increases up to 20-30 % at 70 ºC, which is in agreement with an expected higher beta sheet content at basic solvent conditions and high temperatures.

Previous CD experiments on holo-myoglobin had shown partial unfolding of the protein at 65 ºC, total unfolding at 84.5 ± 1.0 °C (Fändrich et al., 2003), with no indication of an alpha to beta transition at 55 ºC at pH 9.

X-ray Scattering

Figure 3 shows the WAXS curve of ApoMb in D_2O buffer at pH 9 depending on temperature from 25 °C to 65 °C. As reported previously (Hirai et al., 2004), we can discuss about the details of the structural transition of ApoMb in its different hierarchical levels that are the quaternary and tertiary structures (q < ~0.2 $Å^{-1}$), the inter-domain correlation and the intra-domain structure (q = ~0.25–0.8 $Å^{-1}$), and the secondary structure and the closely packed side chains in the hydrophobic cores (q = ~1.1–1.9 $Å^{-1}$), respectively. The arrows plotted in Figure 3 indicate the typical features of the scattering curve of an amyloid formation. Namely, the peak at q = ~1.35 $Å^{-1}$, the broad peak at q = ~0.60 $Å^{-1}$, and the shoulder at q = ~0.09 $Å^{-1}$ correspond to the α-to-β transition (cross-β structure), the pleated sheet stacking, and the oligomerization of denatured ApoMb, respectively. These features appear simultaneously and are evidently seen above ~55 °C. It should be mentioned that the positions of the peaks at q = ~1.35 $Å^{-1}$ and ~0.60 $Å^{-1}$ are slightly larger than those observed in H_2O buffer (Onai et al., 2007). This suggests that in D_2O solvent the formed stacking of the cross-β is more compact as compared with that in H_2O solvent.

Neutron Scattering

The total intensity analysis of the IN13 data at pH9 is shown in Figure 4. The curves for ApoMb and HoloMb both show a 'transition' at about 55 ºC indicative of a more resilient (smaller rate of decrease with temperature) structure at the higher temperature. The CD data showed that in the case of ApoMb, the effect is correlated with the α-to-β transition. In holo-myoglobin, the more resilient state seems to be associated with partial unfolding of the protein and aggregation in accordance with Faendrich et al. (2003). We present only summed elastic intensities as these are more precise to identify dynamical transitions (Reat et al., 2000). Calculation of mean square displacements

gave errors bars that were to big to make any conclusion about a dynamical transition around 55°C.

As the temperature is increased and before total unfolding at higher temperatures, both ApoMb and holo-myoglobin display a transition to a more resilient aggregated dynamic structure at about 55º C. In the case of ApoMb at pH 9, the structure is the β-structure associated with amyloid fibre formation. While in the case of holo-myglobin, the structure is likely due to partial unfolding and an undefined aggregate state (Faendrich et al., 2003). The transition to a more resilient high temperature dynamics structure at ~55º C was also observed in IN13 experiments on ApoMb at pH 7 (data not shown), suggesting that like for holo-myoglobin, ApoMb at pH 7 partially unfolds and aggregates in this pre-transition to total unfolding. In order to explore further the dynamic behaviour of aggregated protein, a temperature scan of the elastic intensity was performed on heat-denatured ApoMb on the IRIS spectrometer at ISIS (Figure 5). The 17 μeV energy resolution condition chosen on the instrument corresponds to a time scale of 50 ps. Interestingly, the data showed a dynamical transition for the sample at about 200 K, similar to that observed in hydrated native holo-myoglobin (0.38g D_2O/ 1 g protein) by Doster et al. (Doster et al., 1989), indicating that the transition is also a property of heat-denatured and aggregated protein. The force constant <k> and the effective force constant <k'> were determined for the low temperature range below 200 K with <k>=3.7 +/- 0.3 N/m and above 200 K with <k'>= 0.17 +/- 0.01 N/m (Zaccai, 2000). In the 100 ps time range values of <k>=3 N/m and <k'>=0.3 N/m were obtained for hydrated holo-myoglobin powder (Zaccai, 2000). At low temperatures the force constants <k> between apo- and holo-myoglobin are similar. Above the dynamical transition temperature, the effective force constant <k'> of apo-myoglobin is significantly smaller than that of holo-myoglobin. The higher degree of hydration of the

apo-myoglobin sample might facilitate protein motion and be the cause of this discrepancy.

Conclusion

In this paper, apo-myglobin (ApoMb) was used as a model biochemical system in order to explore the molecular changes of dynamics in the α-to-β transition associated with amyloid fibril formation. Circular dichroism and neutron scattering experiment were performed on the protein, at pH 9 in H_2O and D_2O, in a wide temperature range. Similar experiments were carried out on holo-myoglobin, on the same solvent condition. Wide angle X-ray scattering was used to confirm the occurrence of the α-to-β transition of ApoMb in D_2O solution at pD 9 by the appeareance of characteristic Bragg peaks. Circular dichroism experiments emphasized that the transition is present above 55°C and only at pH and pD 9 in dilute solution. Apo-myoglobin results show a significant change in molecular dynamics at the α-to-β transition at about 55˚ C, indicating a more *resilient* high temperature β structure phase. The behaviour of the holo-protein as function of temperature is associated with partial unfolding and protein aggregation.

Acknowledgements

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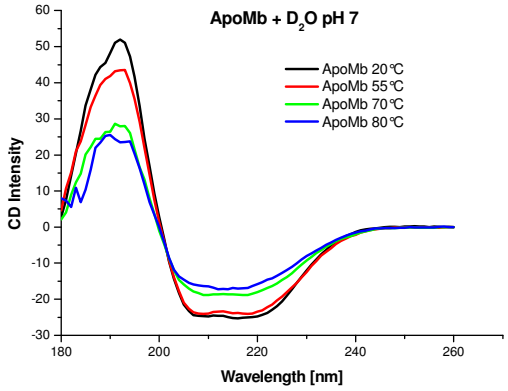
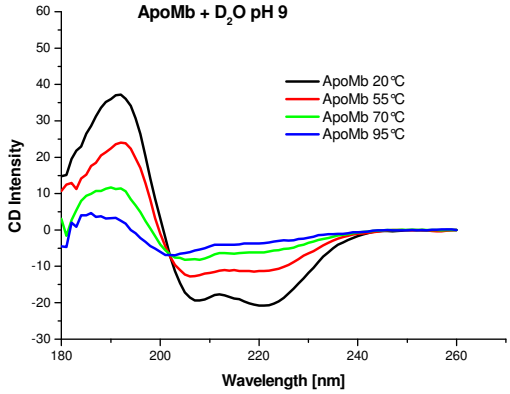
Figures

Figure 1 Circular dichroism data for apo-myoglobin (A) in D_2O at pH 7, (B) in D_2O at pH 9, (C) in H_2O at pH 7 and (D) in H_2O at pH 9 in the temperature range between 20 °C and 95 °C.

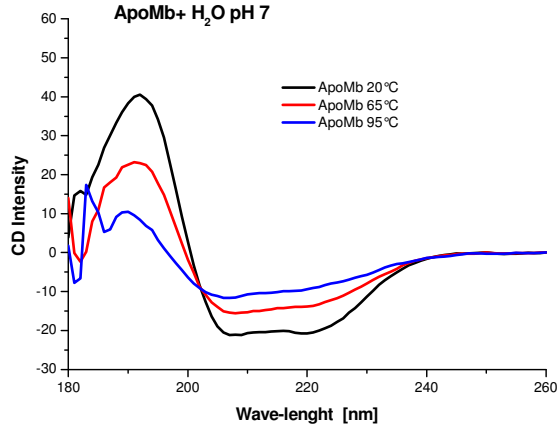
(A)



(B)



(C)



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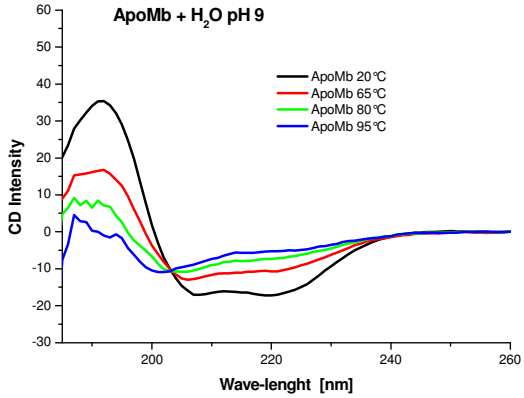


Figure 2 Analysis of the structural content of ApoMb at pH 7 and pH 9 in (A) H_2O and (B) D_2O.

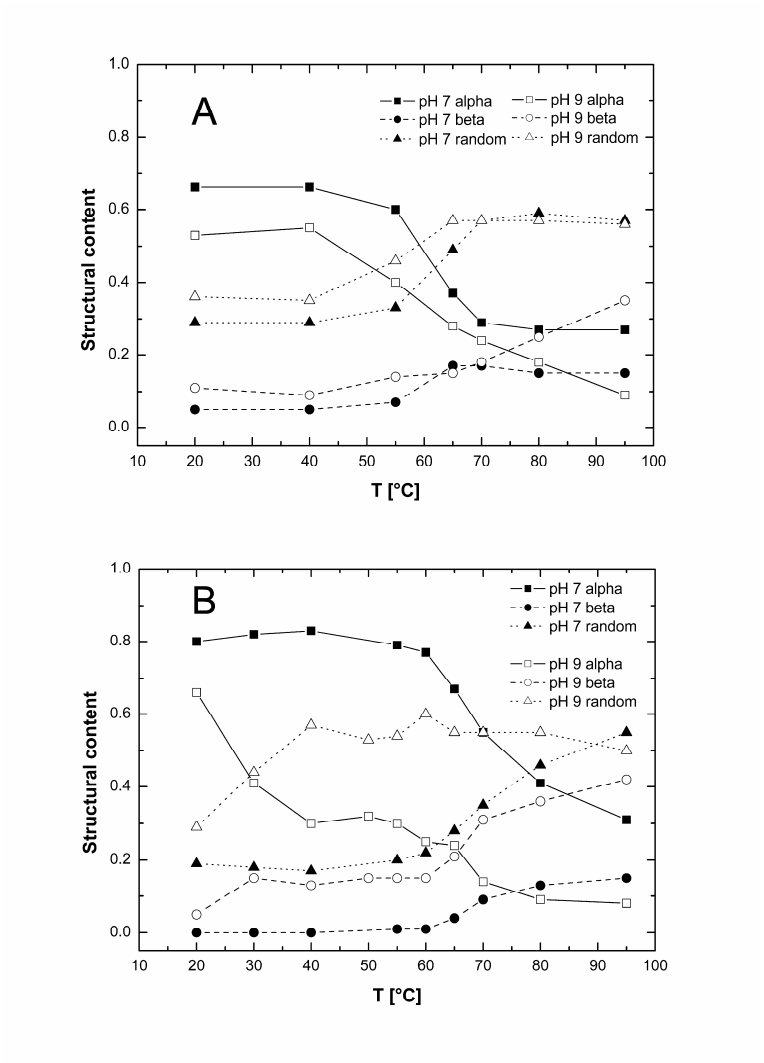


Figure 3. Wide-angle X-ray scattering curve of apo-myoglobin in D_2O solvent at pH 9 in the temperature range from 25 to 65 °C. The arrows (the peaks at q = = ~1.35 Å^{-1} and ~0.60 Å^{-1}, the shoulder at q = ~0.09 Å^{-1}) indicate typical features of amyloid transition. This transition occurred above ~55 °C.

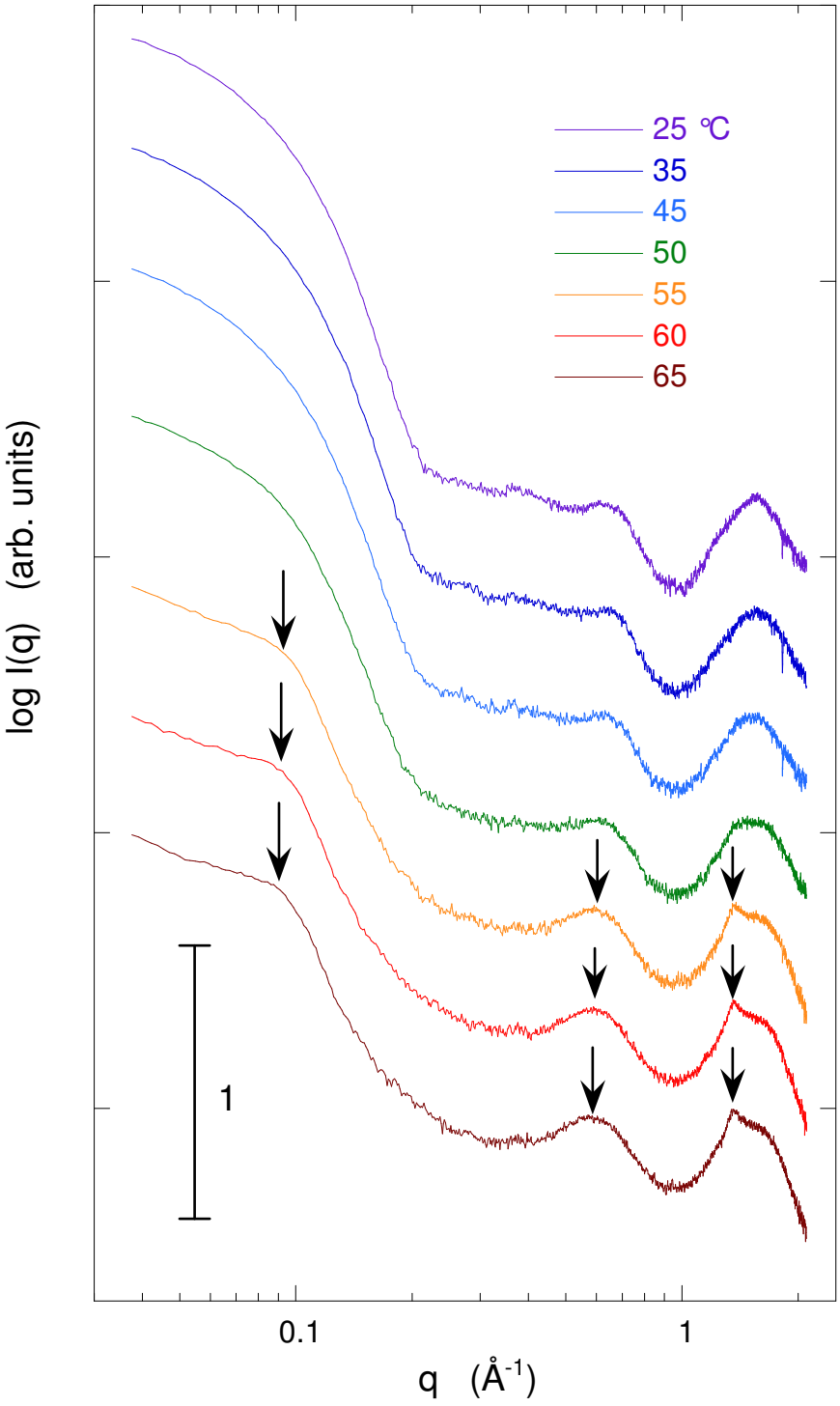


Figure 4. IN13 sum of intensities vs temperature for apo-myoglobin and holo-myoglobin. The broken line is a linear fit for holo-myoglobin and the straight line is a linear fit to apo-myoglobin. Due to limitations of the temperature control, no experimental points could be measured between around 45-55°C.

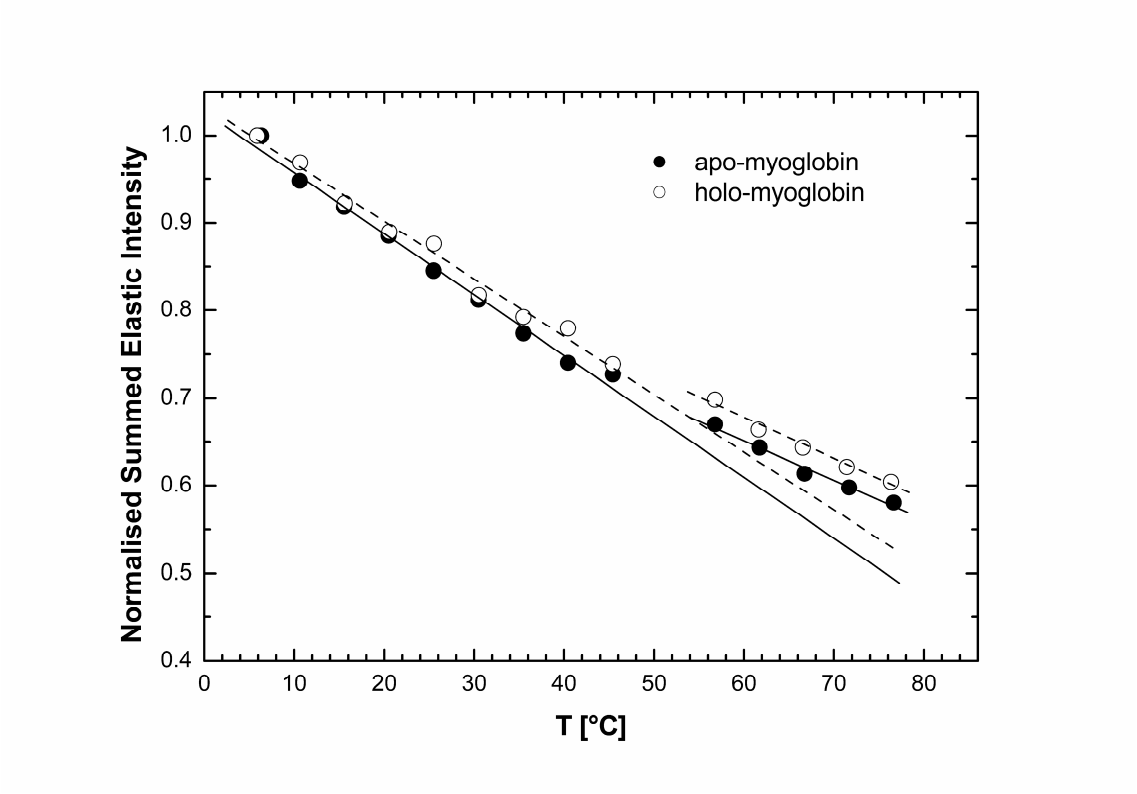
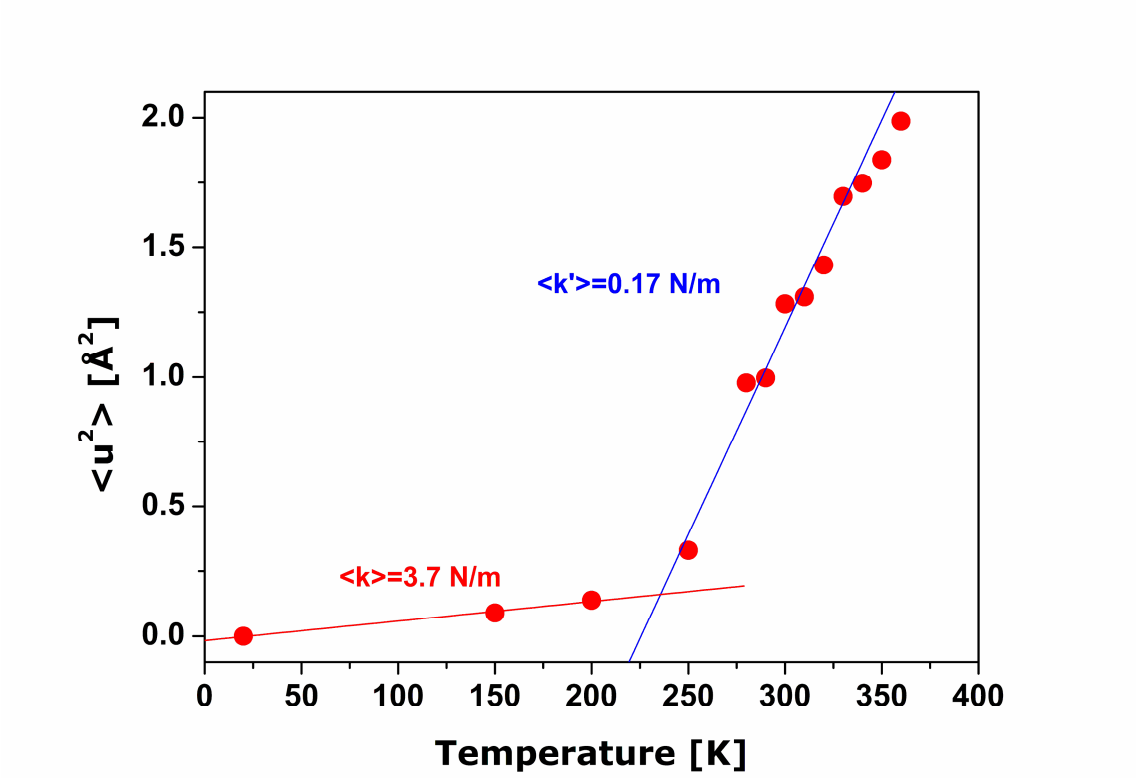


Figure 5. Mean square displacement $\langle u^2 \rangle$ vs temperature from IRIS elastic scan measurements on heat-denatured and aggregated ApoMb. Error bars are within the symbols.



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